Su(var) genes regulate the balance between euchromatin and heterochromatin in *Drosophila*

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Histone lysine methylation is an epigenetic mark to index chromosomal subdomains. In *Drosophila*, H3-K9 di- and trimethylation is mainly controlled by the heterochromatic SU(VAR)3-9 HMTase, a major regulator of position-effect variegation (PEV). In contrast, H3-K27 methylation states are independently mediated by the Pc-group enzyme E(Z). Isolation of 19 point mutants demonstrates that the silencing potential of *Su(var)3-9* increases with its associated HMTase activity. A hyperactive *Su(var)3-9* mutant, *pitkin^D*, displays extensive H3-K9 di- and trimethylation within but also outside pericentric heterochromatin. Notably, mutations in a novel *Su(var)* gene, *Su(var)3-1*, severely restrict *Su(var)3-9*-mediated gene silencing. *Su(var)3-1* was identified as "antimorphic" mutants of the euchromatic H3-S10 kinase JIL-1. *JIL-1^{Su(var)3-1}* mutants maintain kinase activity and do not detectably impair repressive histone lysine methylation marks. However, analyses with seven different PEV rearrangements demonstrate a general role of *JIL-1^{Su(var)3-1}* in controlling heterochromatin compaction and expansion. Our data provide evidence for a dynamic balance between heterochromatin and euchromatin, and define two distinct mechanisms for *Su(var)* gene function. Whereas the majority of *Su(var)*s encode inherent components of heterochromatin that can establish repressive chromatin structures [intrinsic *Su(var)*s], *Su(var)3-1* reflects gain-of-function mutants of a euchromatic component that antagonize the expansion of heterochromatic subdomains [acquired *Su(var)*s].

[**Keywords:** *Su(var)* genes; *Su(var)3-9*; JIL-1; heterochromatin; position-effect variegation; histone lysine methylation]

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Control of higher-order chromatin structure is important for epigenetic gene regulation and functional differentiation of chromosomal domains during development. In *Drosophila* and *Schizosaccharomyces pombe*, genetic dissection of heterochromatic gene silencing identified fundamental mechanisms controlling DNA packaging with histones and other chromosomal proteins into repressive chromatin structures (Reuter and Spierer 1992; Allshire et al. 1994). In *Drosophila*, >50 *Suppressor of position-effect variegation* [*Su(var)*] loci exist, of which ~15 have been molecularly defined. *Su(var)* genes encode structural components of heterochromatin, such as the zinc finger protein *Su(var)3-7* (Delattre et al. 2000; Jaquet et al. 2002) and the chromo domain protein HP1 (Eissen-

berg et al. 1990; Eissenberg and Elgin 2000), but also enzymes that can modify histone N termini (tails), such as the histone deacetylase HDAC1 (DeRubertis et al. 1996; Mottus et al. 2000; Czermin et al. 2001) and the histone methyltransferase (HMTase) *Su(var)3-9* (Rea et al. 2000; Schotta et al. 2002). SU(VAR)3-9, HP1, and SU(VAR)3-7 are inherent components of heterochromatin. In a genetic hierarchy, *Su(var)3-9* is dominant over the other two genes indicating an important role for histone lysine methylation in heterochromatin formation (Schotta et al. 2002).

Histone lysine methylation can be present in mono-, di-, or trimethylation states (Santos-Rosa et al. 2002; Peters et al. 2003), and combinations of active (H3-K4, H3-K36, H3-K79) or repressive (H3-K9, H3-K27, and H4-K20) modifications can index distinct chromosomal subdomains. Hallmarks of constitutive heterochromatin in mammals are H3-K9 trimethylation, H3-K27 monomethylation (Peters et al. 2003; Rice et al. 2003), and H4-K20 trimethylation (Schotta et al. 2004). The major

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HMTases for H3-K9 trimethylation at pericentric heterochromatin are Suv39h enzymes (Peters et al. 2003). Ezh2 is the major H3-K27 HMTase to index the inactive X-chromosome (Xi) and Pc-dependent gene silencing (Plath et al. 2003; Silva et al. 2003); however, it is not known whether Ezh2 contributes to pericentric H3-K27 monomethylation in mammals.

In *Drosophila*, H3-K9 dimethylation was found to be enriched at heterochromatin (Schotta et al. 2002). The development of highly specific antibodies that can discriminate mono-, di-, and trimethylation of H3-K9 versus H3-K27 (Peters et al. 2003) now allowed us to characterize all H3-K9 and H3-K27 methylation states in *Drosophila*. Our data reveal that *Su(var)3-9* mainly controls H3-K9 di- and trimethylation at pericentric heterochromatin, whereas all H3-K27 methylation is mediated by *Enhancer of zeste* [*E(z)*]. The identification and characterization of novel point mutants in *Su(var)3-9* that show differential HMTase activities demonstrate that the silencing potential of *Su(var)3-9* is mainly determined by the kinetic properties of the HMTase reaction. Importantly, we identified the PEV modifier *Su(var)3-1* as a key antagonist for *Su(var)3-9*-dependent gene silencing. *Su(var)3-1* mutants are novel alleles of the euchromatic H3-S10 kinase JIL-1 (Wang et al. 2001). In these mutants, H3-S10 phosphorylation is not altered, indicating that the kinase function of JIL-1^{*Su(var)3-1*} is intact. However, condensation of the male X-chromosome and expansion of heterochromatic subdomains are impaired, suggesting that JIL-1^{*Su(var)3-1*} may regulate the balance between euchromatin and heterochromatin. Although JIL-1 is not an intrinsic component of heterochromatin, JIL-1^{*Su(var)3-1*} mutants display the strongest PEV modifier effect and are dominant over *Su(var)3-9*. Our data define *Su(var)3-1* as a novel class of *Su(var)* genes, in which gain-of-function mutants of a euchromatic component antagonize the expansion of heterochromatic subdomains.

Results

H3-K9 and H3-K27 methylation states in Drosophila heterochromatin

To investigate the distribution of distinct H3-K9 and H3-K27 methylation states in *Drosophila* heterochromatin, we used highly specific antibodies that can discriminate mono-, di-, and trimethylation of H3-K9 versus H3-K27 (Peters et al. 2003) in immunofluorescence analyses. Polytene chromosomes allow the mapping of these marks at a very high resolution because they have a clearly discernible chromocenter where all chromosomes are joined. Attached to this chromocenter is the largely heterochromatic fourth chromosome.

In wild-type chromosomes, the prominent mark of the chromocenter and the fourth chromosome is H3-K9 dimethylation (Fig. 1A). In addition, H3-K9 dimethylation is associated with some euchromatic bands and telomeres (Supplementary Fig. S1). H3-K9 monomethylation is also found in the chromocenter (Fig. 1A) and is asso-

ciated with many euchromatic bands (Supplementary Fig. S1). H3-K9 trimethylation shows a very distinct heterochromatic distribution with a strong enrichment in the chromocenter core (Fig. 1A, arrowhead). The chromocenter core region is also marked by CID, a centromere-specific histone variant in *Drosophila* (Henikoff et al. 2000; data not shown), and likely represents centromeric heterochromatin.

In *Su(var)3-9*-null mutants, H3-K9 monomethylation is not significantly altered. In contrast, H3-K9 di- and trimethylation are strongly reduced in the chromocenter but not in the fourth chromosome (Fig. 1A). Notably, the chromocenter core that is enriched for H3-K9 trimethylation in wild-type chromosomes is prominently marked with H3-K9 dimethylation in *Su(var)3-9* mutants (Fig. 1A, arrowhead).

HP1 is an interaction partner of SU(VAR)3-9 and important to retain SU(VAR)3-9 at pericentric heterochromatin (Schotta et al. 2002). To test whether HP1 is involved in the control of *Su(var)3-9*-dependent H3-K9 di- and trimethylation, we analyzed these methyl-marks in *Su(var)2-5* (HP1) mutants. In the chromocenter, H3-K9 mono-, di-, and trimethylation appear unaltered (Fig. 1A); however, there is a dramatic increase in H3-K9 mono- and dimethylation along euchromatic arms (Supplementary Fig. S1).

All three H3-K27 methylation states are present at pericentric heterochromatin. H3-K27 mono- and dimethylation are broadly associated with the chromocenter region, whereas H3-K27 trimethylation displays a strong accumulation at the chromocenter core (Fig. 1B). Additionally, H3-K27 mono- and dimethylation can be detected at almost all bands in euchromatin, whereas only ~100 bands are marked by H3-K27 trimethylation (Supplementary Fig. S2). H3-K27 methylation states are not significantly altered in *Su(var)3-9*-null mutants (Fig. 1B); however, no H3-K27 methylation can be detected in *E(z)*-null mutants (Fig. 1B).

Immunofluorescence analyses and the different abundance of H3-K9 and H3-K27 methylation states were confirmed by mass spectrometric analyses of bulk nuclear extracts from wild-type and mutant larvae (Fig. 1C). Consistent with previous studies (Schotta et al. 2002) demonstrating dispersed localization of SU(VAR)3-9, H3-K9 methylation levels are significantly increased in the HP1-null background.

Together, the data demonstrate that pericentric heterochromatin in *Drosophila* is largely characterized by H3-K9 di- and H3-K27 mono- and dimethylation. Only the chromocenter core displays a focal enrichment for the trimethylated state (H3-K9 and H3-K27 trimethylation). We provide evidence for the existence of *Su(var)3-9*-independent H3-K9 monomethylation at the chromocenter and of additional H3-K9 HMTases that target the fourth chromosome. In contrast, *E(z)* mediates all three H3-K27 methylation states but does not appear to affect H3-K9 methylation. Although *E(Z)* has been detected at a limited number of bands (Carrington and Jones 1996), the high abundance of H3-K27 methylation indicates that *E(Z)* complexes might not reside at all sites after accomplishing the methylation.

Figure 1. Control of H3-K9 and H3-K27 methylation states in *Drosophila* heterochromatin. (A) Distribution of H3-K9 mono-, di-, and trimethylation in the chromocenter region of salivary gland polytene chromosomes from wild-type, *Su(var)3-9*-null, and HP1-null third instar larvae. (B) Distribution of H3-K27 mono-, di-, and trimethylation in the chromocenter region of polytene chromosomes from wild-type and *Su(var)3-9*-null as well as in whole chromosomes of *E(z)*-null third instar larvae. DNA was stained with propidium iodide. Arrows point to the fourth chromosome; arrowheads indicate the chromocenter core region. (C) Comparative mass spectrometric analyses of H3-K9 and H3-K27 mono-, di-, and trimethylation levels in H3 peptide fragments isolated from nuclear extracts of wild-type, *Su(var)3-9*-null, *E(z)*-null, and HP1-null mutant larvae. For each genotype three different extracts were analyzed (error bar indicates standard deviation). The relative level of a particular H3-K9 methylation state is indicated with respect to the sum of all H3-K9 and H3-K27 modifications present in the H3 peptide populations.

The silencing potential of *Su(var)3-9* mutants correlates with their associated H3-K9 HMTase activity

To identify novel regulatory mechanisms for *Su(var)3-9*-dependent gene silencing, we performed EMS mutagenesis experiments and isolated 56 novel suppressor mutants together with 19 *Su(var)3-9* alleles. Sequence analyses of these novel *Su(var)3-9* alleles revealed that most mutants cluster around the SET domain (Fig. 2A; Supplementary Fig. S3). Although the screen was rather saturated, we could not detect mutants in the SU(VAR)3-9 chromo domain.

In the course of these studies we also identified the exceptionally strong PEV enhancer *pitkin*^D (Kuhfittig et al. 2001) as a novel *Su(var)3-9* allele. The *pitkin*^D mutation causes extensive chromatin compaction in early embryos and induces ectopic recruitment of SU(VAR)3-9

with many euchromatic sites (Kuhfittig et al. 2001). P-element-induced male recombination (cf. Materials and Methods) indicated that the *pitkin* locus maps to region 88E, near *Su(var)3-9*. Sequence analysis revealed a point mutation (R529S) within the SU(VAR)3-9 SET domain. To verify that this mutation is, indeed, *pitkin*^D, we analyzed transgenic flies expressing SU(VAR)3-9^{R529S}. After remobilization, four independent lines were selected that display a very strong PEV enhancer effect that was equivalent to three additional *Su(var)3-9* gene copies (data not shown). Since these data classify the *pitkin*^D mutation as a hypermorphic *Su(var)3-9* allele, we propose to rename this mutant *Su(var)3-9*^{ptn}.

Based on their ability to induce *white* gene silencing in the PEV rearrangement *In(1)w^{m4}*, the other *Su(var)3-9* mutants were categorized into null and hypomorphic alleles. One wild-type copy of *Su(var)3-9* fails to induce

white gene repression (the eyes remain red), whereas two wild-type copies partially silence the *white* gene and display the characteristic white mottled phenotype (Fig. 2B). Next, we crossed these two wild-type copies with our novel *Su(var)3-9^{mut}* mutants. In this combination, some mutant alleles could moderately enhance *white* gene silencing and were regarded as hypomorphic mutants (Fig. 2B). In contrast, mutant alleles that did not alter the *In(1)w^{m4}* phenotype were regarded as null alleles (Fig. 2B).

Next, we tested whether the reduced silencing potential of hypomorphic *Su(var)3-9* mutants possibly correlated with their ability to induce H3-K9 di- and trimethylation at pericentric heterochromatin. In the homozygous *Su(var)3-9¹⁷*-null mutant, pericentric H3-K9 dimethylation is nearly absent and H3-K9 trimethylation is entirely lost from the chromocenter (Fig. 2C). All mutants that were classified as null alleles show a similar loss of H3-K9 methylation. In contrast, hypomorphic alleles show slightly elevated H3-K9 di- and trimethylation at the chromocenter (Fig. 2C). Importantly, hypermorphic *Su(var)3-9^{ptn}* induces strong H3-K9 di- and trimethylation at pericentric heterochromatin (Fig. 2C) and, additionally, very prominent signals at many euchromatic sites (Supplementary Fig. S4A). Mass spectrometric analyses of *Su(var)3-9^{ptn}* nuclear extracts further demonstrate that the

overall levels of H3-K9 di- and trimethylation are significantly increased (Supplementary Fig. S4B).

To examine the mutant proteins in a more direct manner, we performed in vitro HMTase assays with 16 different point mutants. All loss-of-function mutants are catalytically inactive, whereas mutant proteins of hypomorphic alleles are less active than wild-type protein (Fig. 2D). *SU(VAR)3-9^{ptn}* displayed a higher HMTase activity (Fig. 2D).

Finally, we characterized reaction kinetics of the wild-type HMTase versus the *SU(VAR)3-9^{ptn}* mutant. In the starting phase of the reaction, *SU(VAR)3-9^{ptn}* methylates approximately twice as many H3 peptides as the wild-type HMTase and reaches a plateau within ~15 min (Fig. 2E). These data characterize *SU(VAR)3-9^{ptn}* as a hyperactive H3-K9 HMTase and strongly suggest that its hypermorphic phenotype (chromatin compaction and enhanced gene silencing) is caused by enhanced enzymatic activity.

Identification of *JIL-1* mutants as novel regulators of PEV-based gene silencing

In our mutant screens to isolate novel *Su(var)3-9* alleles, we also identified other suppressor mutants that impair *Su(var)3-9*-dependent silencing. The strongest of these mutants has been identified as an allele of the previously

Figure 2. *SU(VAR)3-9* HMTase activity correlates with its ability to induce gene silencing. (A) Protein structure of *SU(VAR)3-9* and positions of the molecularly characterized mutations. Point mutations resulting in amino acid exchanges are in red. (B) Genetic classification of *Su(var)3-9* mutants. *Su(var)3-9^{mut}* alleles were crossed to two extra *Su(var)3-9* copies. Null mutants show the characteristic white mottled phenotype, whereas hypomorphic alleles partially enhance *white* repression. *Su(var)3-9^{ptn}* is a hypermorphic mutant resulting in complete *white* and partial silencing of the *roughest* gene. (C) H3-K9 di- and trimethylation in chromocenter regions of heterozygous *Su(var)3-9¹⁷/Su(var)3-9^{mut}* mutants. Null mutants [exemplified by *Su(var)3-9¹⁰*] show the same loss of H3-K9 di- and trimethylation as the *Su(var)3-9¹⁷/Su(var)3-9¹⁷*-null mutant. H3-K9 methylation levels are elevated in hypomorphic mutants [exemplified by *Su(var)3-9²²*]. Very high levels of H3-K9 di- and trimethylation are found in hypermorphic *Su(var)3-9^{ptn}*. Arrowheads indicate the chromocenter core. (D) In vitro HMTase activity of recombinant *SU(VAR)3-9* mutant proteins. The amount of incorporated label was measured by scintillation counting. (E) Time-course analysis of HMTase reactions with wild-type (wt) and *SU(VAR)3-9^{ptn}* proteins. Incorporation of the radioactive label was measured after different timepoints by scintillation counting.

described *Su(var)3-1* gene (Wustmann et al. 1989). *Su(var)3-1* mutants strongly suppress gene silencing in the *In(1)w^{m4}* PEV rearrangement (Fig. 3A, left panel) and even counteract gene repression that is caused by overexpression of prominent regulators of heterochromatin formation, such as *Su(var)3-9*, *Su(var)2-5* (HP1), and *Su(var)3-7* (Fig. 3A, right panels). Using male recombination, we mapped the *Su(var)3-1* locus to a small region comprising four genes (*Mocs1*, *CG7839*, *CG6302*, and *JIL-1*) between the two P-element insertions *P{EP}EP3688* and *P{EP}EP3657*. Sequencing of candidate open reading frames (ORFs) in all six *Su(var)3-1* alleles revealed nonsense mutations in the C-terminal part of *JIL-1* that would generate truncated proteins (Fig. 3B). We therefore propose naming these mutants *JIL-1^{Su(var)3-1}*.

JIL-1 is a kinase that controls H3-S10 phosphorylation (pH3S10) in interphase nuclei (Wang et al. 2001). In *JIL-1*-null mutants, euchromatic pH3S10 is impaired, whereas mitotic pH3S10 is not altered. We analyzed pH3S10 in polytene chromosomes of *JIL-1^{Su(var)3-1}* mutants by immunofluorescence using a pH3S10-specific antibody. In wild-type chromosomes, pH3S10 is associated with many interbands along chromosomal arms. In *JIL-1^{Su(var)3-1}* mutants, we did not observe a significant change of this pattern (Fig. 3C), indicating that the kinase function of the truncated *JIL-1^{Su(var)3-1}* protein is intact.

JIL-1-null mutants are characterized by condensation of polytene chromosomes, an effect that is most apparent for the male X-chromosome (Wang et al. 2001). To test for changes in the polytene chromosome structure of

JIL-1^{Su(var)3-1} mutants, we analyzed chromosomal squashes of wild-type versus homozygous mutant larvae. Strikingly, the X-chromosome is largely decondensed in *JIL-1^{Su(var)3-1}* males (Fig. 3D). The identity of this chromosome was confirmed by immunofluorescence stainings for MOF, a protein that is specifically associated with the male X-chromosome (Fig. 3D). These data show that truncated *JIL-1^{Su(var)3-1}* acts against the wild-type protein and classify *JIL-1^{Su(var)3-1}* mutants as antimorphic *JIL-1* alleles.

JIL-1^{Su(var)3-1} prevents heterochromatin expansion over a large chromosomal domain

In order to test whether the altered chromosomal structure in *JIL-1^{Su(var)3-1}* mutants would coincide with changes in the pattern of repressive histone lysine methylation marks, we performed IF analyses of polytene chromosomes from wild-type versus mutant larvae. In homozygous *JIL-1^{Su(var)3-1}* mutants, no obvious differences in the patterns of H3-K9 and H3-K27 methylation states or of H4-K20 trimethylation have been detected (Supplementary Fig. S5), which was confirmed by mass spectrometric analyses of nuclear extracts (data not shown).

One possibility to explain the strong derepression of the *white* gene in *In(1)w^{m4}* by *JIL-1^{Su(var)3-1}* could be a shift in the expansion of the heterochromatic structure. We analyzed the effect of *JIL-1^{Su(var)3-1}* mutants on heterochromatin expansion in the PEV rearrangement *T(1;4)w^{m258-21}*, in which heterochromatinization of a large

Figure 3. *Su(var)3-1* mutants are antimorphic alleles of the *JIL-1* kinase. (A) Genomic extra copies of *Su(var)3-9*, *Su(var)2-5*, and *Su(var)3-7* display enhanced silencing of the *white* gene in *In(1)w^{m4}* (upper row), which is impaired in the presence of the strong PEV suppressor mutant *Su(var)3-1* (lower row). (B) Protein structure of *JIL-1*. All mutational lesions in *Su(var)3-1* alleles result in a C-terminally truncated *JIL-1* protein. (S/T) Serine/threonine kinase domain; (S/T/Y) Serine/threonine/tyrosine kinase domain. (C) Polytene chromosomes of wild-type and *Su(var)3-1* mutant larvae were stained with pH3S10-specific antibodies. DNA was stained with propidium iodide. (D) X-chromosome decondensation phenotype of *Su(var)3-1* mutants. In male homozygous mutant larvae, the X-chromosome is largely decondensed and partially loses its banding structure. The arrowhead points to the X-chromosome. The affected chromosome shows association with MOF, a specific marker for the male X-chromosome. DNA was stained with propidium iodide. Bar, 10 μm.

euchromatic region can be monitored. $T(1;4)w^{m258-21}$ is a complex rearrangement in which a part of the X-chromosome (cytological region 3C–3E) comprising ~1 Mb of DNA is brought near to pericentric heterochromatin (Fig. 4A). Because of a translocation of the fourth chromosome, this region can specifically be detected in polytene chromosome spreads. It contains several genes, of which inactivation of *Notch* (clipped wings) and *white* (white eyes) can be monitored phenotypically. In the presence of two extra *Su(var)3-9* copies, the whole region becomes highly compacted and prominently marked by H3-K9 dimethylation (Fig. 4B, bracket, left panel). The repressed state of this region can also be detected by inactivation of *Notch* and, to a lesser extent, of the more distant *white* gene (Fig. 4B, left panel). Notably, heterochromatin does not expand along the fourth chromosome, which is modified by different HMTase complexes that might be critical to the spreading effect. Next, we crossed the two extra copies of *Su(var)3-9* with $JIL-1^{Su(var)3-1}$ and examined the polytene structure at $T(1;4)w^{m258-21}$. Surprisingly, the whole region that was inactivated in the presence of the two extra *Su(var)3-9* copies is not compacted and shows a normal banding pattern (Fig. 4B, right panel). Derepression of this region is further supported by reactivation of the *Notch* and *white* marker genes (Fig. 4B, right panel). These data illustrate that *Su(var)3-9*-mediated expansion of heterochromatin can be impaired by truncated $JIL-1^{Su(var)3-1}$, suggesting an important function for the C terminus of $JIL-1$ in regulating heterochromatin formation.

The compaction of a large chromosomal domain in $T(1;4)w^{m258-21}$ allowed us to examine distinct contributions of H3-K9 di- and trimethylation to heterochromatin expansion. The active and decondensed region that displays a normal banding pattern does not show significant signals for H3-K9 di- or trimethylation (Fig. 4B, bracket, right panel). In contrast, *Su(var)3-9*-induced condensation of this chromosomal region is accompanied by strong H3-K9 dimethylation; however, we could not detect significant H3-K9 trimethylation (Fig. 4B, bracket, left panel). These data demonstrate that in *Drosophila*, H3-K9 dimethylation is the major mark to induce chromatin condensation and expansion of heterochromatic structures. H3-K9 trimethylation plays a minor role for heterochromatin formation, and the strong enrichment for this mark in the chromocenter core rather supports the argument for a function of H3-K9 trimethylation in organizing chromatin structure at centromeres.

JIL-1^{Su(var)3-1} generally antagonizes Su(var)3-9 gene function at PEV epialleles

In order to test whether $JIL-1^{Su(var)3-1}$ generally impairs *Su(var)3-9*-mediated heterochromatin expansion, we tested the effect of $JIL-1^{Su(var)3-1}$ mutants on seven different PEV rearrangements. In the first class of rearrangements, chromosomal aberrations bring an endogenous gene locus (marker gene) near to pericentric heterochromatin (Table 1). In wild type, these marker genes are largely silenced, detectable by white mottled eyes

Figure 4. *Su(var)3-1* mutants impair expansion of heterochromatin. (A) Translocation $T(1;4)w^{m258-21}$ contains the X-chromosomal region 3C–3E comprising ~1 Mb of DNA, which can be detected because of contacts to the fourth chromosome. Heterochromatin expansion inactivates the two marker genes *Notch* (clipped wings) and *white* (white eyes). (B) Immunocytological analysis of H3-K9 di- and trimethylation at $T(1;4)w^{m258-21}$. (Left panel) In the presence of two extra *Su(var)3-9* copies, region 3C–3E becomes compacted (bracket) and marked with H3-K9 dimethylation but not H3-K9 trimethylation. Transcriptional repression is indicated by a large number of *Notch* wings (51%) and white mottled sector eyes (41%). (Right panel) The presence of *Su(var)3-1* mutations impairs expansion of heterochromatin (bracket), resulting in a normal banding pattern, loss of H3-K9 dimethylation, and restored transcriptional activity as indicated by wild-type (*Notch*⁺) wings (91%) and red (*white*⁺) eyes (95%).

Table 1. *JIL-1^{Su(var)3-1}* generally antagonizes *Su(var)3-9* mediated heterochromatin expansion

PEV epialleles	Marker gene(s)	Chr.	Phenotype when marker is fully expressed	% wild-type expression of marker gene in		
				P{3-9} +/+	Su(var)3-1; +/+	Su(var)3-1; P{3-9} +/+
Class I						
<i>In(1)w^{m4}</i>	<i>white</i>	X	Red eyes	0%	100%	95%
<i>Dp(1;f)γ238</i>	<i>yellow</i>	X	Black abdominal pigmentation	6%	70%	45%
<i>T(2;3)Sb^V</i>	<i>stubble</i>	3	Short bristles	2%	64%	n.d.
<i>T(1;4)w^{m258-21}</i>	<i>Notch</i>	X	Clipped wings	49%	100%	91%
	<i>white</i>		Red eyes	41%	100%	95%
Class II						
<i>In(3L)BL1</i>	<i>lacZ</i> -transgene	3	<i>lacZ</i> expression	26%	73%	60%
	<i>w⁺</i>		Red eyes	33%	86%	58%
<i>Trp(3;Y)BL2</i>	<i>lacZ</i> -transgene	Y	<i>lacZ</i> expression	26%	68%	53%
	<i>w⁺</i>		Red eyes	25%	98%	68%
<i>T(2;3)V21^e</i>	tandem repeats	3	Red eyes	22%	85%	n.d.
<i>PlacW(92E)</i>	of <i>w⁺</i> transgene					

Expression of marker genes in different PEV epialleles was monitored in the presence of two *Su(var)3-9* extra copies [*P[3-9] +/+*], *JIL-1^{Su(var)3-1}* mutants [*Su(var)3-1 +/+*], and the combination of two *Su(var)3-9* extra copies with *JIL-1^{Su(var)3-1}* mutants [*Su(var)3-1; P[3-9] +/+*]. Expression of the respective marker genes was evaluated according to Materials and Methods. Representative phenotypes are depicted in Supplementary Figure 6.

[*In(1)w^{m4}*], yellow abdomen with few black spots [*Dp(1;f)γ238*], or long thoracic bristles due to inactivation of the dominant *Sb^V* mutant [*T(2;3)Sb^V*], respectively. *JIL-1^{Su(var)3-1}* can release silencing of these marker genes and even antagonizes *Su(var)3-9*-mediated gene silencing (Table 1; Supplementary Fig. S6).

In the second class of PEV rearrangements, transgene insertions containing *lacZ* and *white* marker genes are brought near to heterochromatic regions (Table 1). In these rearrangements we have analyzed expression of marker genes in different developmental stages, *lacZ* expression in larval salivary gland tissues, and *white* expression in adult flies. Both *lacZ* and *white* are silenced by overexpression of *Su(var)3-9*, but strongly derepressed in the presence of *JIL-1^{Su(var)3-1}* mutants (Table 1; Supplementary Fig. S6). Also in these rearrangements, *Su(var)3-9*-mediated expansion of heterochromatin is restricted by *JIL-1^{Su(var)3-1}*, which is indicated by reactivation of both marker genes in flies containing genomic extra copies of *Su(var)3-9* together with *JIL1^{Su(var)3-1}* mutants.

These data demonstrate that *JIL-1^{Su(var)3-1}* antagonizes *Su(var)3-9*-mediated heterochromatin expansion at different genomic regions and developmental stages. We therefore conclude that *JIL-1^{Su(var)3-1}* is a general regulator for the balance between euchromatin and heterochromatin.

Discussion

Su(var) genes as inherent components of heterochromatin

The identification of *Su(var)* genes in *Drosophila* revealed many important proteins regulating higher-order chromatin structure. *SU(VAR)3-9*, *HP1*, and *SU(VAR)3-7* are inherent components of heterochromatin that can

establish and maintain a heterochromatic chromatin structure. By genetic means, *Su(var)3-9* is the dominant component over *Su(var)2-5* (*HP1*) and *Su(var)3-7* (Schotta et al. 2002), indicating a major function of the associated HMTase in the formation of repressive chromatin regions.

In *Drosophila*, heterochromatic gene silencing is mainly determined by H3-K9 dimethylation. In agreement with its in vitro HMTase activity (Eskeland et al. 2004), *Su(var)3-9* regulates H3-K9 di- and trimethylation at pericentric heterochromatin. In addition, other H3-K9-specific HMTases must exist that mediate H3-K9 dimethylation at the fourth chromosome and in the chromocenter core of *Su(var)3-9* mutants. *HP1* is another important control factor for H3-K9 methylation because in *Su(var)2-5* mutants, H3-K9 mono- and dimethylation are dramatically increased along chromosomal arms. Although *SU(VAR)3-9* shows extensive association with heterochromatic and euchromatic regions in *HP1*-null cells, the shift in these methylation patterns could also be mediated by other H3-K9-specific HMTases. At pericentric heterochromatin, H3-K9 methylation states appear unaltered; however, a slight reduction of H3-K9 dimethylation that is not detectable by immunofluorescence might be the cause of the *HP1 Su(var)* effect. Alternatively, impairment of H4-K20 trimethylation at pericentric heterochromatin (Schotta et al. 2004) could cause the loss of silencing in *HP1* mutants.

The analysis of *Su(var)3-9* mutants revealed novel hypomorphic and null alleles that show differential effects on H3-K9 methylation states. A direct correlation between their HMTase activities and the amount of gene silencing demonstrates that establishment of heterochromatic structures is to a large extent determined by the kinetic properties of the *SU(VAR)3-9* HMTase reaction. Consistently, hyperactive *SU(VAR)3-9^{ptm}* in-

duces enhanced H3-K9 di- and trimethylation, concomitant with an expansion of heterochromatin. This results in severe phenotypes such as dominant female sterility caused by overcompaction of the whole chromatin in early embryos (Kuhfittig et al. 2001). In this study we show that *Su(var)3-9^{ptn}* mutants display around 100–200 additional bands with enhanced H3-K9 di- and trimethylation in the arms of polytene chromosomes. These bands likely correspond to endogenous SU(VAR)3-9-binding sites (Greil et al. 2003) and demonstrate that hyperactive SU(VAR)3-9^{ptn} can mediate the expansion of repressive structures also at chromosomal arms.

A loss-of-function *Su(var)* mutant reduces gene silencing by removing one or several intrinsic components of heterochromatin. *Su(var)3-9^{ptn}* induces enhanced gene silencing, and it was therefore surprising to encode a mutant of a “classical” *Su(var)* gene. Based on our functional characterization, we identified this mutant as the first hypermorphic (gain-of-function) allele of an intrinsic *Su(var)* gene. Many more PEV modifier mutants that cause enhanced gene silencing have been isolated. Molecular characterization of some of these genes revealed components of active chromatin, such as transcription factors (Dorn et al. 1993); however, further characterization of this class of genes might also uncover other hypermorphic mutants of intrinsic *Su(var)* genes and contribute to the understanding of heterochromatin regulation.

JIL-1^{Su(var)3-1} represents a novel class of PEV modifier mutants with acquired Su(var) function

Our mutant screen to identify genes that impair *Su(var)3-9*-mediated gene silencing led to the molecular identification of *Su(var)3-1*, the strongest *Su(var)* gene to date. *Su(var)3-1* can restrict heterochromatin formation and generally dominate over the strong silencing effect of *Su(var)3-9* extra copies. *Su(var)3-1* carries C-terminal truncations of the H3-S10 kinase JIL-1 that do not affect its ability to phosphorylate H3-S10 but selectively impair the expansion of heterochromatin.

Intrinsic *Su(var)* genes impair or weaken the heterochromatic structure. Loss-of-function *Su(var)3-9* alleles or mutations in HDAC1 reduce H3-K9 dimethylation (Czermin et al. 2001), whereas HP1 mutants impair H4-K20 trimethylation (Schotta et al. 2004) at pericentric heterochromatin. JIL-1 is not an intrinsic component of heterochromatin (Wang et al. 2001) and does not affect any of the known repressive histone lysine methylation marks at pericentric heterochromatin. Nevertheless, *JIL-1^{Su(var)3-1}* alleles display a strong *Su(var)* effect. We therefore categorize *JIL-1^{Su(var)3-1}* as a novel type of mutants with acquired *Su(var)* function. In contrast to intrinsic *Su(var)s* that encode components directly involved in heterochromatin formation, acquired *Su(var)* mutants would antagonize heterochromatin formation by stabilizing the euchromatic state or preventing heterochromatin expansion.

Our data assign a novel function to the C terminus of

the JIL-1 kinase that is independent of its H3-S10 kinase activity. Lack of the C terminus could prevent phosphorylation or interaction with an as-yet-unknown target protein. Alternatively, the C terminus could comprise control functions that prevent excessive phosphorylation of this target. There are many possible target proteins that could be involved in higher-order chromatin formation. For example, the striking X-chromosome decondensation phenotype of *JIL-1^{Su(var)3-1}* mutants has also been described for *iswi* and *nurf301*, subunits of the NURF complex (Deuring et al. 2000; Badenhorst et al. 2002). Particularly, *nurf301* mutants display a dominant *Su(var)* effect, indicating that chromatin remodeling processes are involved in the regulation of heterochromatin. Another possible target of the JIL-1^{Su(var)3-1} kinase might be the histone variant H2A.Z. In budding yeast, H2A.Z is required to prevent spreading of heterochromatin into euchromatin (Meneghini et al. 2003). This protective function might be modulated by JIL-1^{Su(var)3-1}-mediated phosphorylation of H2A.Z. Finally, some intrinsic *Su(var)s* such as SU(VAR)3-9 and HP1 are phosphoproteins (Eissenberg et al. 1994; Aagaard et al. 2000) and JIL-1^{Su(var)3-1}-mediated phosphorylation of those proteins might affect their function in heterochromatin expansion.

The balance between euchromatin and heterochromatin

Based on our data we propose a model for a dynamic balance between euchromatin and heterochromatin (Fig. 5). In PEV rearrangements, the boundary between these two states is determined by the antagonistic functions of euchromatic regulators (e.g., JIL-1) and the SU(VAR)3-9 HMTase that mediates H3-K9 dimethylation, the major mark of heterochromatin (Fig. 5A). The boundary between euchromatin and heterochromatin is not static and depends on the activity and abundance of inherent components of heterochromatin, such as, for example, hyperactive SU(VAR)3-9^{ptn} (Fig. 5B) or overexpression of *Su(var)3-9* (Fig. 5C). In contrast, hypoactive or null mutants of *Su(var)3-9* weaken heterochromatin formation and favor the propagation of the euchromatic state. In *JIL-1^{Su(var)3-1}* mutants, heterochromatin expansion is severely repressed, even under conditions of elevated *Su(var)3-9* function, and is most likely antagonized by a currently unknown proteins that are potential targets of the gain-of-function JIL-1^{Su(var)3-1} kinase. Dependent on the phosphorylation state of these targets, either heterochromatin expansion can be blocked or the propagation of the euchromatic state is highly stabilized (Fig. 5D).

In this work we have significantly extended mechanistic insights into the formation of heterochromatin and discovered *Su(var)3-1* as a novel class of PEV modifiers that controls the balance between euchromatic and heterochromatic subdomains. Because of the dominant role of *JIL-1^{Su(var)3-1}* over major components of heterochromatin [intrinsic *Su(var)s*], the putative JIL-1 mutant targets are predicted to have important functions in gene silencing and the higher-order structuring of chromatin. The ongoing

Figure 5. *Su(var)* genes regulate the balance between euchromatin and heterochromatin. The antagonistic functions of acquired and intrinsic *Su(var)* genes establish a boundary between euchromatin and heterochromatin. Hyperactive *SU(VAR)3-9^{ptn}* (B) or overexpression of *Su(var)3-9* (C) can expand heterochromatin, whereas *JIL-1^{Su(var)3-1}* stabilizes the euchromatic state by acting on an unknown target protein X (D). Please see main text for details.

ing analysis on the full definition of *Su(var)* gene function is therefore likely to reveal both the genetic and molecular hierarchy that dictates epigenetic gene control.

Materials and methods

Drosophila cultures, stocks, and genetic analysis

Flies were reared on standard medium at 25°C. Chromosomes and mutations not noted here are described in FlyBase (<http://flybase.net>). *Su(var)3-9* and *Su(var)3-1* mutations were isolated by their dominant suppressor effect on *white* variegation of *In(1)w^{m4}* in the background of *E(var)* mutations or transgenic copies of *Su(var)3-9* after EMS (2.5 mM) mutagenesis (Reuter et al. 1986; Tschiersch et al. 1994). The effects of *Su(var)3-9* mutations on *white* gene silencing in *w^{m4}* were quantified in transheterozygotes with an additional *P[(ry⁺) Su(var)3-9 11.5kb]100E* genomic copy (Tschiersch et al. 1994). For molecular analysis, genomic DNA of *Su(var)3-9⁰⁶/Su(var)3-9^{9x}* heterozygotes was used to amplify the coding region of the *Su(var)3-9* locus. Due to the DNA fragment inserted into *Su(var)3-9⁰⁶* (Schotta et al. 2002), only PCR products of *Su(var)3-9^{9x}* are generated. Sequence information for all mutant alleles is available at GenBank (AJ290956).

Pitkin^D, originally assigned to region 67C due to a modifier of dominant female sterility was finally mapped by P-transposase-induced male recombination near *Su(var)3-9* with the help of EP elements. *w/Y; CyO, H[w⁺mC = P2-3]HoP2.1/+; ru ptn^D e/EP(3)* males were crossed to *+/+; ru e/ru e* females. Recombinant *+ e/ru e* and *ru +/ru e* flies were tested for the presence of *ptn^D* (PEV

enhancer effect and dominant female sterility) by crosses to *w^{m4}* flies. The full-length *Su(var)3-9* cDNA containing the *ptn^D* mutation was introduced into *pP[GS[ry⁺, hEGFP]]* (Schotta and Reuter 2000). Independent *pP[GS[ry⁺, hs cDNA Su(var)3-9^{ptn} EGFP]]* transgenes were mobilized in crosses with *TM3, ry^{RK} Sb e [P(ry⁺)2-3]99B* and derivative lines with *ptn^D*-specific mutant effects established.

HP1-null *Df(2L)TE128X11/Su(var)2-5⁰⁴* larvae survive up to third instar and were identified as GFP-negative larvae in the progeny of *CyO GFP/Df(2L)TE128X11 × CyO GFP/Su(var)2-5⁰⁴*. *E(z)*-null larvae were generated by a cross of *y; mwh E(z)⁵ red e/TM3, y⁺ Sb Ser × E(z)¹⁵ red/TM3, Sb Ser*. Transheterozygous *mwh E(z)⁵ red e/E(z)¹⁵ red* larvae were identified by their *red* mutant Malpighian tube phenotype.

In vitro histone methyltransferase assays and mass spectrometry analysis

HMTase activities of recombinant GST-tagged proteins were analyzed according to Czermin et al. (2001). For the methyltransferase assays 1 μg of recombinant protein, 1 μg of histone H3 peptide (amino acids 1–20; Upstate Biotech), and 0.5 μL of SAM (1 μCi/mL; Amersham) were incubated for various times at 30°C in 25 μL of HIM buffer (Czermin et al. 2001). Mass spectrometry analysis of reaction products was performed according to Peters et al. (2003).

Nuclear extracts were prepared from wild-type, *SU(VAR)3-9*-null [*Su(var)3-9¹⁷/Su(var)3-9¹⁷*], HP1-null [*Df(2L)TE128X11/Su(var)2-5⁰⁴*], *E(Z)*-null [*E(z)⁵/E(z)¹⁵*], *Su(var)3-9^{ptn}* [*Su(var)3-9^{ptn}/+* and *Su(var)3-9^{ptn}/Su(var)3-9¹⁷*] third instar larvae and separated by 18% SDS-PAGE. Following Coomassie staining, histone H3 bands were excised and processed according to Peters et al. (2003).

Immunostaining of polytene chromosomes

Preparation and staining of polytene chromosomes was performed as described (Schotta et al. 2002). Chromosomes were incubated with rabbit polyclonal α-mono-, α-di-, and α-trimethyl H3-K9 or H3-K27, respectively (1:50–1:100/5% dry milk), α-phospho H3-S10 (Upstate; 1:50/5% dry milk) antibodies at 4°C overnight, followed by incubation with Alexa Fluor 488 conjugated (Molecular Probes) secondary antibody for 2 h at 37°C (1:50/5% dry milk). Preparations were examined with confocal laser scanning microscopy (LSM 510; Zeiss).

Molecular cloning of *Su(var)3-1* and analysis of heterochromatic silencing

Su(var)3-1 was mapped to the genomic region of *JIL-1* at 86A by P-transposase-induced male recombination using a series of *EP(3)* P-element insertions. After a cross of homozygous *se ss* females with *+/Y; CyO, H[w⁺mC = P2-3]HoP2.1/+; se Su(var)3-1⁰² ss e ro/EP(3)* males, *se+* and *+ss* recombinant chromosomes were selected and consecutively tested for the presence of *Su(var)3-1⁰²* by a cross to *w^{m4}* flies. The coding sequence of *JIL-1* was PCR-amplified from genomic DNA of homozygous *Su(var)3-1^{mut}* flies and subjected to sequence analyses.

Suppression of heterochromatization by *Su(var)3-1* was studied in *T(1;4)w^{m258-21}/T(1;4)w^{70126.5}* larvae (Reuter et al. 1982) with an autosomal constitution of either *Su(var)3-1⁰² e ro/P[(ry⁺) Su(var)3-9 11.5kb]96A + P[(ry⁺) Su(var)3-9 11.5kb]100E* or *TM6B, Sb/P[(ry⁺) Su(var)3-9 11.5kb]96A + P[(ry⁺) Su(var)3-9 11.5kb]100E* generated by a cross of *T(1;4)w^{m258-21}/FM1, y^{31d} w^a dm B; Su(var)3-1⁰² e ro/TM6B, Sb* females with *T(1;4)w^{70126.5}/Y; P[(ry⁺) Su(var)3-9 11.5kb]96A + P[(ry⁺) Su(var)3-9 11.5kb]100E*

males. P[ry⁺ *Su(var)3-9* 11.5kb]96A + P[ry⁺ *Su(var)3-9* 11.5kb]100E contains two additional genomic *Su(var)3-9* copies inserted in 96A and 100E. T(1;4)w^{m258-21}/T(1;4)w^{70126.5} and T(1;4)w^{70126.5}/FM1, y^{31d} w^a dm B larvae were distinguished by yellow versus white Malpighian tubes.

The epistatic effect of *Su(var)3-102* on *SU(VAR)3-9*-induced gene silencing was studied in *Dp(1;f)g238* (Le et al. 1995) variegating for *yellow*, T(2;3)V21e1A with repeat-dependent *white* gene silencing (Dorer and Henikoff 1994), T(2;3)BL1, and T(Y;2)BL2, showing P[(w⁺), *HS-lacZ*] transgene silencing (Lu et al. 1998). *Su(var)3-102*-dependent compensation of PEV enhancement was studied in genotypes with two additional genomic *Su(var)3-9* copies (see Supplementary Fig. S6). Expression levels of marker genes were determined by classifying the phenotypes into 5 categories (0 = no expression, 5 = full expression).

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Su(var) genes regulate the balance between euchromatin and heterochromatin in *Drosophila*

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